Investigation of bacterioplankton communities in aquatic karst pools in Bärenschacht cave of Bernese Oberland

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Abstract: Karst subterraneous aquifers are highly diverse in structure and very important in the formation of ground water, which is the main source of freshwater supply for a significant proportion of the world's population. Microorganisms can play an important role in karstification, carbon cycle and element mobility, but so far little is known about the bacteria of aquatic karst ecosystems. In this study, karst pools with differing hydrology in the Bärenschacht cave of the Bernese Oberland, Switzerland were investigated for a period of six months. Two crystalline pools were supplied by dripping water whereas one epiphreatic pool was renewed only by the rising groundwater table at intervals of several days to months. Chemical parameters such as conductivity, pH, ion concentration, as well as bacterial abundance and diversity were determined at several time points. The investigated pools showed remarkably different physicochemical parameters as well as bacterial properties. Although the dominant bacterial group in all three systems was -Proteobacteria, no population overlap inside this group was found between the crystalline pools and the epiphreatic system. Actinobacteria were present mainly in the systems with dripping water supply, whereas bacteria from the Flavobacteriaceae family were identified in both types of systems. Some microorganisms affiliated with Bacteroidetes could also be isolated and investigated in more detail. Generally, most of the identified microorganisms were not closely related to typical freshwater bacteria. Therefore, karst habitats might represent an environment for very specialized microorganisms.

INVESTIGATION OF BACTERIOPLANKTON COMMUNITIES IN AQUATIC KARST POOLS IN BÄRENSCHACHT CAVE OF BERNESE OBERLAND

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Karst subterraneous aquifers are highly diverse in structure and very important in the formation of ground water, which is the main source of freshwater supply for a significant proportion of the world's population. Microorganisms can play an important role in karstification, carbon cycle and element mobility, but so far little is known about the bacteria of aquatic karst ecosystems. In this study, karst pools with differing hydrology in the Bärenschacht cave of the Bernese Oberland, Switzerland were investigated for a period of six months. Two crystalline pools were supplied by dripping water whereas one epiphreatic pool was renewed only by the rising groundwater table at intervals of several days to months. Chemical parameters such as conductivity, pH, ion concentration, as well as bacterial abundance and diversity were determined at several time points. The investigated pools showed remarkably different physicochemical parameters as well as bacterial properties. Although the dominant bacterial group in all three systems was β-*Proteobacteria*, no population overlap inside this group was found between the crystalline pools and the epiphreatic system. *Actinobacteria* were present mainly in the systems with dripping water supply, whereas bacteria from the *Flavobacteriaceae* family were identified in both types of systems. Some microorganisms affiliated with *Bacteroidetes* could also be isolated and investigated in more detail. Generally, most of the identified microorganisms were not closely related to typical freshwater bacteria. Therefore, karst habitats might represent an environment for very specialized microorganisms.

1. Introduction

Ground water represents a major source of freshwater supply for a significant proportion of the world's population, and subterraneous karst aquifers play a vital role in groundwater genesis and initial distribution. Presently, this resource is coming under increasing pressure due to the general recession of glaciers in the context of global warming (Ford and Williams, 2007).

The Bärenschacht cave of the Bernese Oberland (Switzerland) is characterized by an entrance part formed of shafts and steeply dipping galleries of mostly vadose origin which leads into a labyrinth consisting of galleries of mainly phreatic origin. The surface rocks above the cave consist of Globigerina marls and Flysch. Therefore, the water input into Bärenschacht originates almost entirely from the nearby Siebenhengste and Schrattenfluh cave systems that are located in the same catchment area (Häuselmann, 2002). Since Bärenschacht forms the hydrological link between these systems and the springs draining into Lake Thun (Häuselmann, 2002), it allows insight into the genesis and behaviour of an undisturbed deep karst system. Periodic flood events typically result in a rise of the water table in the labyrinthine part of the Bärenschacht cave system. As a consequence, there are numerous relatively small flood-formed pools in this section of the cave with water renewal times ranging from days to years. These epiphreatic pools represent comparatively easily accessible model systems for a better understanding of the chemical and microbiological transformation that may occur in karst ground water at oxic condition.

Microorganisms can play an important role in karstification and they can influence carbon cycle and element mobility (Ford and Williams, 2007, Gabrovsek, *et al*., 2000). So far research about the subsurface microbiota in caves has frequently focused on chemoautotrophy, e.g., their utilization of the available sulphur sources (Macalady, *et al*., 2008, Barton and Luiszer, 2005). By contrast, little is known about the composition of the heteroorganotrophic microbial assemblages, in particular of those planktonic microbes that inhabit undisturbed and fully oxygenized karst water systems.

We studied the abundances and community composition of planktonic microbes in three oxygenated pools from the epiphreatic and vadose zone of Bärenschacht that differed in hydrology and water chemistry. In one of the systems we moreover compared two molecular biological approaches for cultivation-independent community analysis, 16S rRNA sequences analysis and determinative whole-cell fluorescence *in situ* hybridization (FISH).

2. Materials and Methods

Three pools in the labyrinth part of Bärenschacht cave were sampled at least monthly from October 2007 to March 2008. The epiphreatic pool NGIII is located in the Grand Nord gallery; it is approximately 20 m long and 0.5 m deep. The two pools JI and JII are similar in dimensions as NGIII, but differ in hydrology. They are situated in the Jessica gallery of the upper, non-flooded part of the labyrinth, and both JI and JII are exclusively fed by seepage water. Water samples were collected with sterile syringes from a depth of 5 cm. Subsamples for microscopic analysis were fixed with formaldehyde (2% final concentration) at the site. On several occasions (NGIII: December 29, 2007 and February 20, 2008; JI and JII February 25, 2008) unfixed pool water (1.5 to 2.5 l) was filtered onto white polycarbonate filters (type GTTP: 0.2 µm pore size; diameter, 47 mm, Millipore) in the cave to collect microbial biomass for later DNA extraction. The filters were directly placed into sterile tubes containing lysis buffer of the PowerSoil DNA Isolation Kit (MOBIO laboratories) for transport. All samples were delivered to the lab within 24 h after collection.

A data logger was placed on the bottom of the NGIII pool to monitor water temperature and water level. Conductivity and pH were measured after return to the laboratory with an InoLab benchtop meter (WTW) and a pH-Vision 6071 microcomputer (Jenco Electronics), respectively. Thirty ml of water sample was frozen for late determination of total organic carbon (TOC) on a TOC-5000 analyzer (Shimadzu). Nitrate concentrations in pool water were measured photometrically (cadmium reduction method) (Wood, *et al*., 1967) after filtration through membrane filters (type GTTP, Millipore) that had been pre-rinsed with 2 M HCl and deionised sterile water (Milli-Q, Millipore). The concentrations of sulphate, chloride and of cations were assessed by ion chromatography (Compact IC 761, Metrohm).

Bacterial abundances in pool NGIII were determined from formaldehyde-fixed samples. Portions of 2 to 5 ml were stained with 4'-6-diamidino-2-phenylindole (DAPI, final concentration, 6.7 mg ml^{-1}) (Porter and Feig, 1980) and filtered onto black polycarbonate filters (pore size, 0.22 mm, diameter, 25 mm, Osmonics). At least 1000 bacteria per sample were counted by epifluorescence microscopy (AxioImager.M1, filter set 01, Carl Zeiss) at 1000X magnification. Five to 7 ml of formaldehyde-fixed samples from pool NGIII were filtered onto white polycarbonate filters (type GTTP, 0.2 µm pore size; diameter, 47 mm, Millipore), air-dried and stored at -20° C for later processing. Staining by FISH and catalyzed reporter deposition (CARD) was performed as described previously (Sekar, *et al*., 2003). Horseradish peroxidase labelled oligonucleotide probes were applied that were targeted to all Bacteria (EUB I-III) (Daims, *et al*., 1999), β-*Proteobacteria* (BET42a), members of the Cytophaga-Flavobacteria lineage of Bacteroidetes (CF319a), α-*Proteobacteria* (ALF968), γ-Proteobacteria (GAM42a), and *Actinobacteria* (HGC69a) (Amann, *et al*., 1995). Tyramides custom labelled with the fluorescent dye Alexa488 (Invitrogen) were used for signal amplification. The hybridized filters were counterstained with DAPI (1 mg ml^{-1}) and the fractions of hybridized cells were determined in 500–1000 DAPI-stained cells by epifluorescence microscopy.

Biomass for DNA extraction was collected from JI and JII on Feb 25, 2008. NGIII biomass samples from two time points were pooled (December 29, 2007 and February 20, 2008). DNA was extracted with the PowerSoil DNA Isolation Kit (MOBIO laboratories). Primers GM3F and GM4R (Muyzer, *et al*., 1995) were used for the amplification of 16S rRNA genes. The purified PCR products (QIAquick PCR purification kit, QIAGEN) were cloned into E. coli (TOPO TA cloning kit, Invitrogen) and insert-bearing plasmids were prepared with the QIAprep Spin Miniprep Kit (QUIAGEN). Sequencing of 16S rRNA genes with primers M13R, M13F (Messing, 1983) and GM1 F (Muyzer, *et al*., 1993) was carried out on an ABI 3130x Genetic Analyzer using the ABI BigDye chemistry (Applied Biosystems). Partial sequences were assembled (Vector NTI, Invitrogen) and checked fro chimeric origin by the software Pintail (Ashelford, *et al*., 2005). Phylogenetic analyses were performed with the ARB software package (Ludwig, *et al*., 2004).

Cave water samples were plated on three types of media: YST (0.5g starch, 0.5 g yeast extract, 0.5 g peptone), PCA (Merck) and PCA/10. Two dilution factors were used: 10^{-1} ml and 10^{-2} ml. The plates were incubated first for 1 to 3 days at 18° C, and then for several weeks at 8° C. Isolated strains were weekly subcultured and identified by sequencing of their 16S rRNA genes.

3. Results and Discussion

Water temperature and pH were rather similar in all three pools and did not substantially vary during the study period (6.4 to 6.8° C and 7.6 to 7.8, respectively). Four flooding events were recorded, with maximal levels of the water table between 3 m (March 1–3, 2008) and 16 m (October 5–6, 2007) (Fig. 1). The effect of periodic flooding on the water chemistry of the epiphreatic NGIII pool was reflected in higher variability of TOC concentrations and conductivity,

as well as in changes in NO_3 concentrations in time periods separated by flooding events (e.g., 1.2 mg l^{-1} in October/November 2007 vs. 2.2 mg l^{-1} in December 2007/January 2008). TOC concentration in NGIII (3.2 ± 0.9 mg l^{-1}, mean ± 1 standard deviation) significantly exceeded those of the two crystalline pools (JI: 0.7 ± 0.3 mg l^{-1}; JII: 0.5 ± 0.4 mg l^{-1}), whereas the opposite was true for conductivity (NGIII: 190 ± 20 µS cm^{-1}; JI: 345 ± 5 µS cm^{-1}; JII: 360 ± 2.5 µS cm^{-1}). Concentrations of NO_3 and Ca^{2+} were substantially higher in pool JI (5.46 ± 0.23 mg l^{-1}, and 72 ± 1 mg l^{-1}) than in the other two study systems (JII: 2.89 ± 0.17 mg l^{-1} and 57 ± 1 mg l^{-1}; NGIII: 1.8 ± 0.7 mg l^{-1} and 41 ± 4 mg l^{-1}). By contrast, JII featured substantially higher concentrations of SO_4^{2-} and Mg^{2+} than the other two pools.

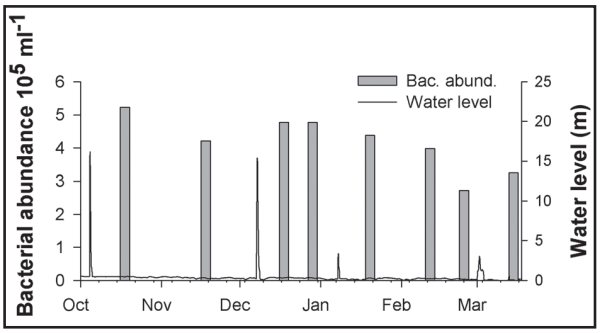

Figure 1: Changes in water level and of total bacterial abundance observed at the NGIII pool of Bärenschacht cave between October 2007 and March 2008.

Bacterial abundances in 8 samples from pool NGIII ranged between 2.7×10^5 ml^{-1} and 5.2×10^5 ml^{-1} (Fig. 1). Total cell numbers tended to declined with time if the period between successive flooding events exceeded one month (October to November 2007 and January to March 2008). This indicates that besides a growth arrest due to substrate limitation mortality by protistan predation or viral lysis might have affected the microbial communities (Pernthaler, 2005). However, a microscopic inspection of several samples suggested that the abundances of free-living phagotrophic flagellates were very low (data not shown).

No quantitative microscopic analysis of bacteria on membrane filters was possible in samples from JI and JII, because (i) microbial cell numbers in the other two pools were extremely low (<10^4 cells ml^{-1}) and (ii) the water from these systems contained high numbers of inorganic particles that impeded the evaluation of more concentrated samples. Therefore, no precise determination of bacterial abundances could be achieved. In order to quantify microbes from crystalline subsurface water pools it might be necessary to separate cells from particles by density gradient centrifugation (Fazi, *et al.*, 2005), or to perform analyses by flow cytometry (Hammes, *et al.*, 2008). However, the low cell numbers in JI and JII might pose a problem even for these approaches.

Created libraries of bacterial 16S rRNA genes consisted of 102 clones for NGIII, 108 for JI, and 103 for JII. A large fraction of sequence types from NGIII and JII (43 and 45%, respectively) was related to β-*Proteobacteria*, but <15% of all sequences from JI. A more detailed analysis of the phylogenetic affiliation of β-*Proteobacteria* revealed striking differences between the three systems (Fig. 2): While *Methylophilaceae* represented the dominant group in NGIII, these bacteria were entirely absent in the other two habitats. Bacteria from this lineage have been found in high abundances in the sub-to anoxic layers of a mesotrophic lake, whereas they were rare in oxygenated waters (Salcher, et al., 2008). Most β-*Proteobacteria* in the pools from the vadose zone were affiliated with either *Comomonadaceae* (JI) or *Oxalobacteriaceae* (JII). β-*Proteobacteria* are known to be a major component of bacterioplankton in many surface freshwater systems (Glöckner, *et al.*, 1999). However, none of the sequence types from the studied pools was closely related to the typical lineages that are known from lakes or rivers, suggesting that subsurface karst pools may harbour a highly specialized planktonic microflora. This conclusion is further corroborated by the phylogenetic affiliation of other sequence types in our libraries (data not shown). For example, several sequences from NGIII and JII fell into the TM7 and OP10 phyla, respectively, that are not known to occur in surface freshwater habitats (Zwart, *et al.*, 2002). The crystalline pool with highest NO_3 concentration (JI) harboured several phylotypes affiliated with *Nitrospira*, a group of nitrite oxidizing bacteria known from soils (Roesch, *et al.*, 2007) and nitrifying biofilms (Daims, *et al.*, 2001). These bacteria were

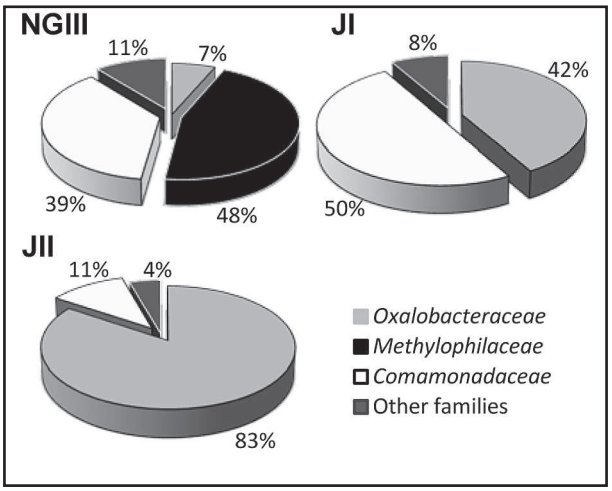

Figure 2: Phylogenetic affiliation of 16SrRNA gene sequences of β-Proteobacteria in the three studied pools NGIII, JI, and JII.

also detected in karst spring water samples during periods of low input of agriculturally influenced surface water (Pronk, *et al*., 2009). Interestingly, some phylotypes affiliated with *Bacteroidetes* were present both in the clone libraries and in the culture collection, which is in contrast to findings from surface waters (Alonso, *et al*., 2007).

The detection rates of bacteria in NGIII by CARD-FISH varied from <40% of total cell counts in October/November 2007 to >60% in January/February 2008 (mean, 54 ± 12%). This lies within the range of values reported for more productive surface freshwater systems (Posch, *et al*., 2009). Only between 49 % and 75 % of all hybridized *Bacteria* could be further identified by the set of group-specific probes, confirming the presence of other bacterial lineages as indicated by sequence analysis. β-*Proteobacteria* represented the most abundant bacterial group that could be identified by FISH in all samples from NGIII (48 ± 7% of *Bacteria*), followed by microbes affiliated with the *Cytophaga-Flavobacteria* lineage of *Bacteroidetes* (CFB, 8 ± 2% of *Bacteria*). By contrast the relative abundances of α-*Proteobacteria* never exceeded 6% of hybridized cells, and *Actinobacteria* could only be detected in 2 out of 8 samples. Figure 3 compares the fractions of sequence types affiliated with different bacterial taxa in the clone library from NGIII with the proportions of these groups detected by FISH on the same sampling time points. This allows an assessment of the bias that is potentially introduced by a preferential PCR amplification of particular sequence types (von Wintzingerode, *et al*., 1997). A clear overrepresentation of phylotypes affiliated with CFB was observed. By contrast, a somewhat higher fraction of β-*Proteobacteria* was found in pool water by direct microscopic inspection than by cloning of 16S rRNA genes. Besides differential amplification by PCR it is conceivable that CFB might have featured higher numbers of rRNA operons than other bacteria. Bacteria affiliated with CFB formed the most prominent cultivable bacterial group from NGIII, and it has been shown that isolates with higher rRNA operon number tend to form colonies on solid media more rapidly than others (Klappenbach, *et al*., 2000).

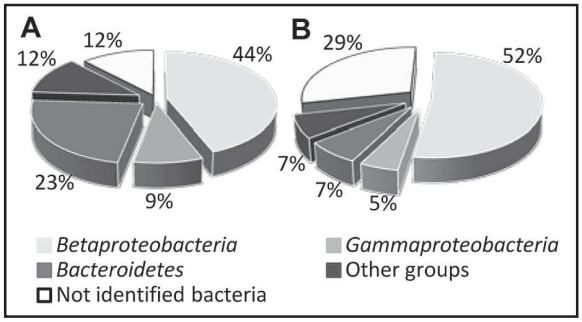

Figure 3:. Community composition of Bacteria in pool NGIII according to phylogenetic analysis of 16S rRNA gene sequences (A) or microscopic assessment after CARD-FISH staining (B).

4. Conclusions

Three fully oxygenated subsurface karst pools with contrasting hydrogeology and chemistry were found to harbor diverse microbial assemblages. β-*Proteobacteria* constituted the most prominent bacterial lineage in all three systems, but each habitat featured a set of distinct 16S rRNA phylotypes affiliated with this group. Moreover, the fine-scale phylogenetic composition of the microbial assemblages in the studied pools clearly differed from surface water communities. Therefore, subsurface karst pools appear to feature a highly specialized planktonic microflora. However, a direct microscopic analysis of bacterial community composition in subsurface karst pools with long water residence times may be hampered by both high background of particulate matter and low cell numbers.

5. References:

ALONSO, C., F. WARNECKE, R. AMANN, and J. PERNTHALER (2007) High local and global diversity of Flavobacteria in marine plankton. Environmental Microbiology 9, 1253–1266.

AMANN, R.I., W. LUDWIG, and K.H. SCHLEIFER (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiological Reviews 59, 143–169.

ASHELFORD, K.E., N.A. CHUZHANOVA, J.C. FRY, A.J. JONES, and A.J. WEIGHTMAN (2005) At least 1 in 20 16S rRNA sequence records currently held in public repositories is estimated to contain substantial anomalies. Applied and Environmental Microbiology 71, 7724–7736.

BARTON, H.A. and F. LUISZER (2005) Microbial metabolic structure in a sulfidic cave hot spring: Potential mechanisms of biospeleogenesis. Journal of Cave and Karst Studies 67, 28–38.

DAIMS, H., A. BRUHL, R. AMANN, K.H. SCHLEIFER, and M. WAGNER (1999) The domain–specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. Systematic and Applied Microbiology 22, 434–444.

DAIMS, H., J.L. NIELSEN, P.H. NIELSEN, K.H. SCHLEIFER, and M. WAGNER (2001) In situ characterization of Nitrospira–like nitrite oxidizing bacteria active in wastewater treatment plants. Applied and Environmental Microbiology 67, 5273–5284.

FAZI, S., S. AMALFITANO, J. PERNTHALER, and A. PUDDU (2005) Bacterial communities associated with benthic organic matter in headwater stream microhabitats. Environmental Microbiology 7, 1633–1640.

Ford, D.C. and P. Willams (2007) Karst Hydrogeology and Geomorphology. John Wiley & Sons, London, 576 p.2nd ed.

GABROVSEK, F., B. MENNE, and W. DREYBRODT (2000) A model of early evolution of karst conduits affected by subterranean CO2 sources. Environmental Geology 39, 531–543.

GLÖCKNER, F.O., B.M. FUCHS, and R. AMANN (1999) Bacterioplankton compositions of lakes and oceans: a first comparison based on fluorescence in situ hybridization. Applied and Environmental Microbiology 65, 3721–3726.

HAMMES, F., M. BERNEY, Y.Y. WANG, M. VITAL, O. KOSTER, and T. EGLI (2008) Flow–cytometric total bacterial cell counts as a descriptive microbiological parameter for drinking water treatment processes. Water Research 42, 269–277.

Häuselmann, P. (2002) Cave genesis and its relationship to surface processes: Investigations in the Siebenhengste region (BE, Switzerland). PhD thesis, University of Fribourg, Fribourg.

KLAPPENBACH, J.A., J.M. DUNBAR, and T.M. SCHMIDT (2000) rRNA operon copy number reflects ecological strategies of bacteria. Applied and Environmental Microbiology 66, 1328–1333.

LUDWIG, W., O. STRUNK, R. WESTRAM, L. RICHTER, H. MEIER, YADHUKUMAR, A. BUCHNER, T. LAI, S. STEPPI, et al. (2004) ARB: a software environment for sequence data. Nucleic Acids Research 32, 1363–1371.

MACALADY, J.L., S. DATTAGUPTA, I. SCHAPERDOTH, D.S. JONES, G.K. DRUSCHEL, and D. EASTMAN (2008) Niche differentiation among sulfur–oxidizing bacterial populations in cave waters. Isme Journal 2, 590–601.

MESSING, J. (1983) New M13 Vectors for Cloning. Methods in Enzymology 101, 20–78.

MUYZER, G., A. TESKE, C.O. WIRSEN, and H.W. JANNASCH (1995) Phylogenetic relationship of Thiomicrospira species and their identification in deep–sea hydrothermal ventes by denaturing gradient gel electrophoresis of 16S rDNA fragments. Archives of Microbiology 164, 165–172.

MUYZER, G., E.C. DE WAAL, and A.G. UITTERLINDEN (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction–amplified genes coding for 16S rRNA. Applied and Environmental Microbiology 59, 695–700.

PERNTHALER, J. (2005) Predation on prokaryotes in the water column and its ecological implications. Nature Reviews Microbiology 3, 537–546.

PORTER, K.G. and Y.S. FEIG (1980) The use of DAPI for identifying and counting aquatic microflora. Limnology and Oceanography 25, 943–948.

POSCH, T., J. FRANZOI, M. PRADER, and M. M. SALCHER (2009) New image analysis tool to study biomass and morphotypes of three major bacterioplankton groups in an alpine lake. Aquatic Microbial Ecology 54, in press.

PRONK, M., N. GOLDSCHEIDER, and J. ZOPFI (2009) Microbial communities in karst groundwater and their potential use for biomonitoring. Hydrogeology Journal 17, 37–48.

ROESCH, L.F., R.R. FULTHORPE, A. RIVA, G. CASELLA, A.K.M. HADWIN, A.D. KENT, S.H. DAROUB, F.A.O. CAMARGO, W.G. FARMERIE, et al. (2007) Pyrosequencing enumerates and contrasts soil microbial diversity. Isme Journal 1, 283–290.

SALCHER, M.M., J. PERNTHALER, M. ZEDER, R. PSENNER, and T. POSCH (2008) Spatio–temporal niche separation of planktonic Betaproteobacteria in an oligo–mesotrophic lake. Environmental Microbiology 10, 2074–2086.

SEKAR, R., A. PERNTHALER, J. PERNTHALER, F. WARNECKE, T. POSCH, and R. AMANN (2003) An improved protocol for the quantification of freshwater actinobacteria by fluorescence in situ hybridization. Applied and Environmental Microbiology 69, 2928–2935.

VON WINTZINGERODE, F., U.B. GÖBEL, and E. STACKEBRANDT (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR–based rRNA analysis. FEMS Microbiology Reviews 21, 213–229.

WOOD, E.D., F.A.J. ARMSTRONG, and F.A. RICHARDS (1967) Determination of Nitrate in Sea Water by Cadmium–Copper Reduction to Nitrite. Journal of the Marine Biological Association of the United Kingdom 47, 23.

ZWART, G., B.C. CRUMP, M. AGTERVELD, F. HAGEN, and S.K. HAN (2002) Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. Aquatic Microbial Ecology 28, 141–155.